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DNA BARCODES OF CLOSELY RELATED (BUT MORPHOLOGICALLY AND ECOLOGICALLY
DISTINCT) SPECIES OF SKIPPER BUTTERFLIES (HESPERIIDAE) CAN DIFFER
BY ONLY ONE TO THREE NUCLEOTIDES

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ABSTRACT. Unlike most species of Lepidoptera whose DNA barcodes have been examined, closely related taxa in each of three pairs of hesperids (*Polyctor cleta* and *P. polyctor*, *Cobalus virbins* and *C. fidicula*, *Neoxeniades luda* and *N. pluviasilva* Burns, new species) seem indistinguishable by their barcodes; but that is when some of the cytochrome *c* oxidase I (COI) sequences are short and sample sizes are small. These skipper butterflies are unquestionably distinct species, as evidenced by genitalic and facies differences and by ecologic segregation, i.e., one species of each pair in dry forest, the other in adjacent rain forest in Area de Conservación Guanacaste in northwestern Costa Rica. This national park is the source of the specimens used in this study, all of which were reared. Larval foodplants are of no or problematic value in distinguishing these species. Large samples of individuals whose barcodes are acceptably long reveal slight interspecific differentiation (involving just one to three nucleotides) in all three pairs of skippers. Clearly, the chronic practice of various taxonomists of setting arbitrary levels of differentiation for delimiting species is unrealistic.

Additional key words: Area de Conservación Guanacaste, Costa Rica, dry forest, rain forest, foodplants, genitalia, *Neoxeniades pluviasilva* Burns, n. sp.

A DNA barcode is the base pair (bp) sequence of a short (~650 bp), standard segment of the genome (Hebert *et al.* 2003). In animals, this is part of the mitochondrial gene cytochrome *c* oxidase I (COI). Because the COI gene generally mutates at evolutionarily rapid rates, comparison of barcodes in a sample of individuals best reveals differentiation at low taxonomic levels. Hence barcodes can be extremely useful in distinguishing and identifying species. Coupling this concept with the idea of always comparing the same short length of COI across a wide diversity of taxonomic groups—and doing so with demonstrable success—is what led to the catchy name “DNA barcodes” (Hebert *et al.* 2003). Even though COI had been used effectively in various evolutionary

and taxonomic studies at and around the species level well before this epithet appeared, in the few years since its introduction, barcodes have been used for their specific purpose with notable results and with rapidly increasing frequency.

The rearing of myriad wild-caught caterpillars in Area de Conservación Guanacaste (ACG) in northwestern Costa Rica is now approaching its thirtieth year (for information about both site and rearing process, see Miller *et al.* 2006, Janzen & Hallwachs 2006, Burns & Janzen 2001). DNA barcodes (of a total of 4,260 reared adults) have been able to distinguish among almost 98% of 521 previously known species of the lepidopteran families Hesperidae (skipper butterflies), Sphingidae (sphinx moths), and Saturniidae (wild silk moths)

(Hajibabaei *et al.* 2006, Janzen *et al.* 2005). Rare cases where barcodes failed, which always involved closely related congeners, are worth examining in more detail. In this paper we treat three such pairs of congeneric skipper species (noted in Hajibabaei *et al.* 2006:table 1). We map the ecologic separation of the species in each pair in and very near ACG. We document the species status of each member of a pair (and describe one as new) on morphologic grounds. We discuss the various degrees to which larval diets, although specialized, are unreliable for species discrimination. And we show, by scrutinizing sequence length and composition, that DNA barcodes separate the species after all. Despite the immense value of DNA barcodes and the fact that they have often indicated overlooked species, it is important to consider characters besides the barcodes themselves—a point made repeatedly in the revelation of 10 cryptic species hiding under the one name *Astraptes fulgerator* (Walch) in ACG (Hebert *et al.* 2004).

THE SPECIES PAIRS IN QUESTION

Ecologic separation (Figs. 1-3). Each pair comprises a dry-forest species and a rainforest species. Parapatry of this kind is a recurrent distribution pattern among closely related lepidopteran species in ACG. In each pair of the following list, the dry-forest species comes first: *Polyctor cleta* Evans and *P. polyctor* (Prittwitz), *Cobalus virbius* (Cramer) and *C. fidicula* (Hewitson), *Neoxeniades luda* (Hewitson) and *N. pluviasilva* Burns (a new species described below). *Polyctor* is a pyrgine genus, and *Cobalus* and *Neoxeniades* are hesperiine genera.

From our distribution data, parapatry in both pairs of hesperiine species appears to be complete (Figs. 2, 3) whereas that in the pyrgine pair does not (Fig. 1). Out of 211 reared individuals (wild-caught as caterpillars) of the rainforest species *P. polyctor*, four came from dry forest. The genitalia of these apparent strays have been KOH-dissected and thoroughly studied to be sure of

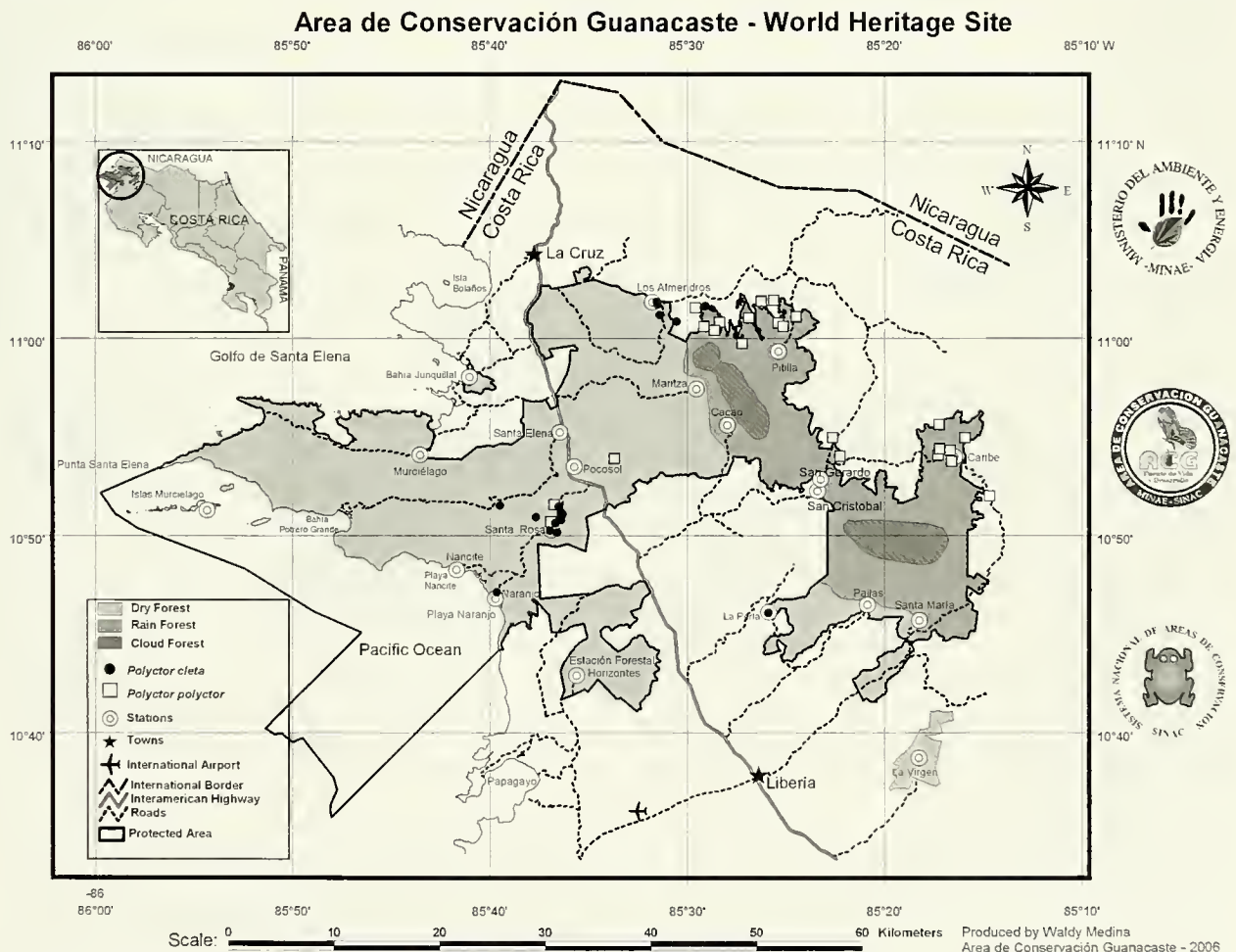


FIG. 1. Spatial distribution of *Polyctor cleta* and *P. polyctor* in and near ACG.

their specific determination. Because both species of this essentially parapatric pair of *Polyctor* eat the same three species of foodplants (Table 3), and because one of these plants occurs in both rain and dry forest, a female wandering from rain forest can find an attractive foodplant in dry forest and oviposit on it. The flight of these skippers is far stronger than necessary to travel the distance involved. Of the four *P. polyctor* caterpillars found in dry forest, three were eating the species of foodplant most often eaten by this skipper in rain forest (and because two of those were found on the very same plant, they are probably offspring of a single female); the fourth caterpillar was eating an exceedingly common, but strictly dry-forest, species that is by far the preferred foodplant of *P. cleta*.

A small number of *P. cleta* caterpillars found in disturbed ecotone between dry and rain forest, and less than 2 km from the latter, were eating the main foodplant of *P. polyctor*. KOH-dissection and examination of the genitalia of the four adults reared

from this group gave no hint of hybridization.

Morphologic differences (Figs. 4-41; Tables 1, 2).

In all three pairs, the brown ground color of the adult averages paler in the dry-forest species than it does in its rainforest counterpart (Figs. 4-27). This is especially evident when comparing long series of more or less recently reared (therefore unfaded) specimens.

In both sexes of *Polyctor*, a spot spanning the distal end of the forewing cell is hyaline in *P. cleta* but opaque in *P. polyctor*. A male secondary sex character in these species of *Polyctor* comprises a tuft of long hairlike scales arising near the base of the dorsal hindwing costa, as well as an elongate patch of pale specialized scales embraced by the swollen beginning of vein 7 and a similarly swollen, closely adjacent length of vein 6; in both veins, swelling extends out to the end of the cell; and the hairlike scales are long enough to overlie the special patch. These presumably pheromone-disseminating hairs are mostly to entirely dark in *P. cleta* but pale (often orangish) in *P. polyctor* (cf. Figs. 4 and 6).

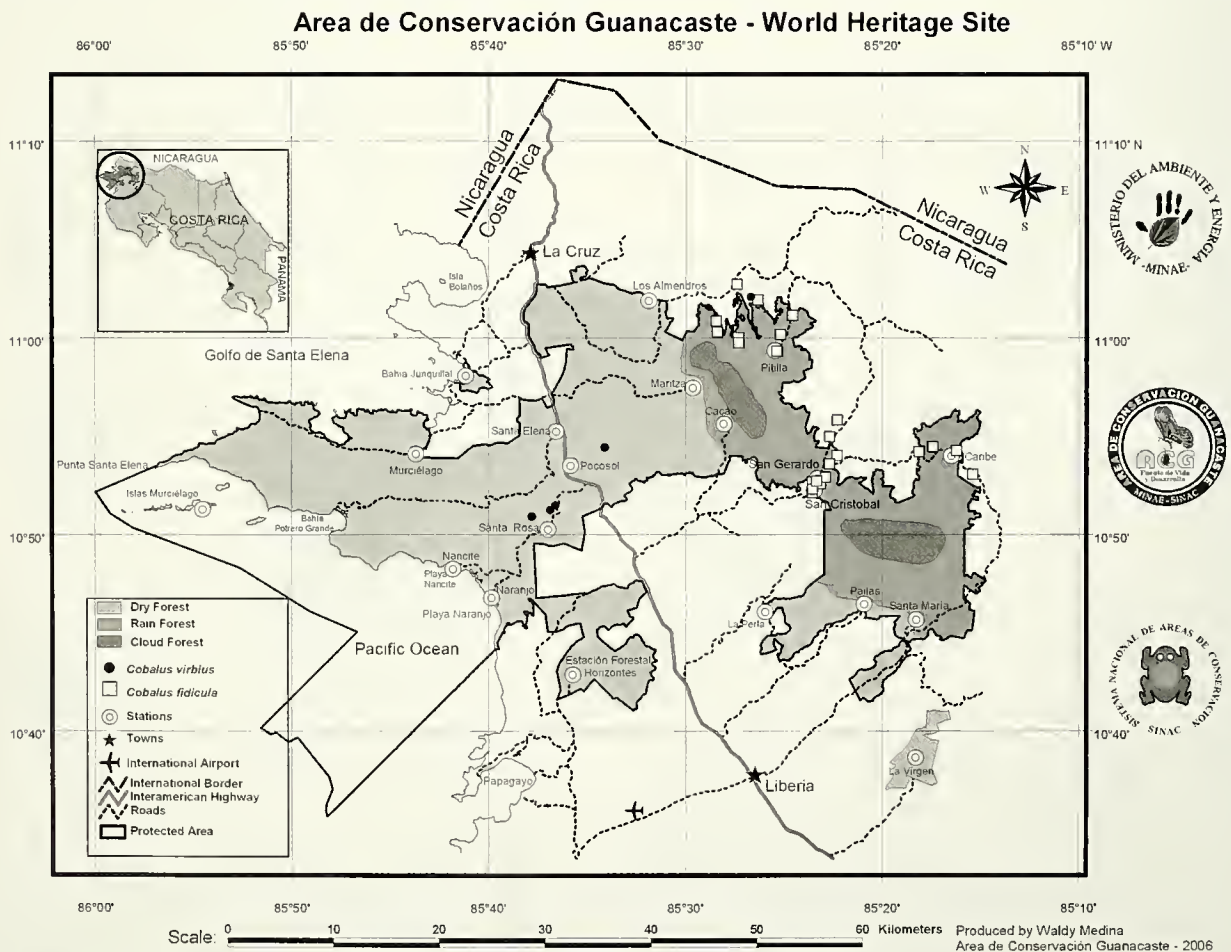


FIG. 2. Spatial distribution of *Cobalus virbius* and *C. fidicula* in and near ACG.

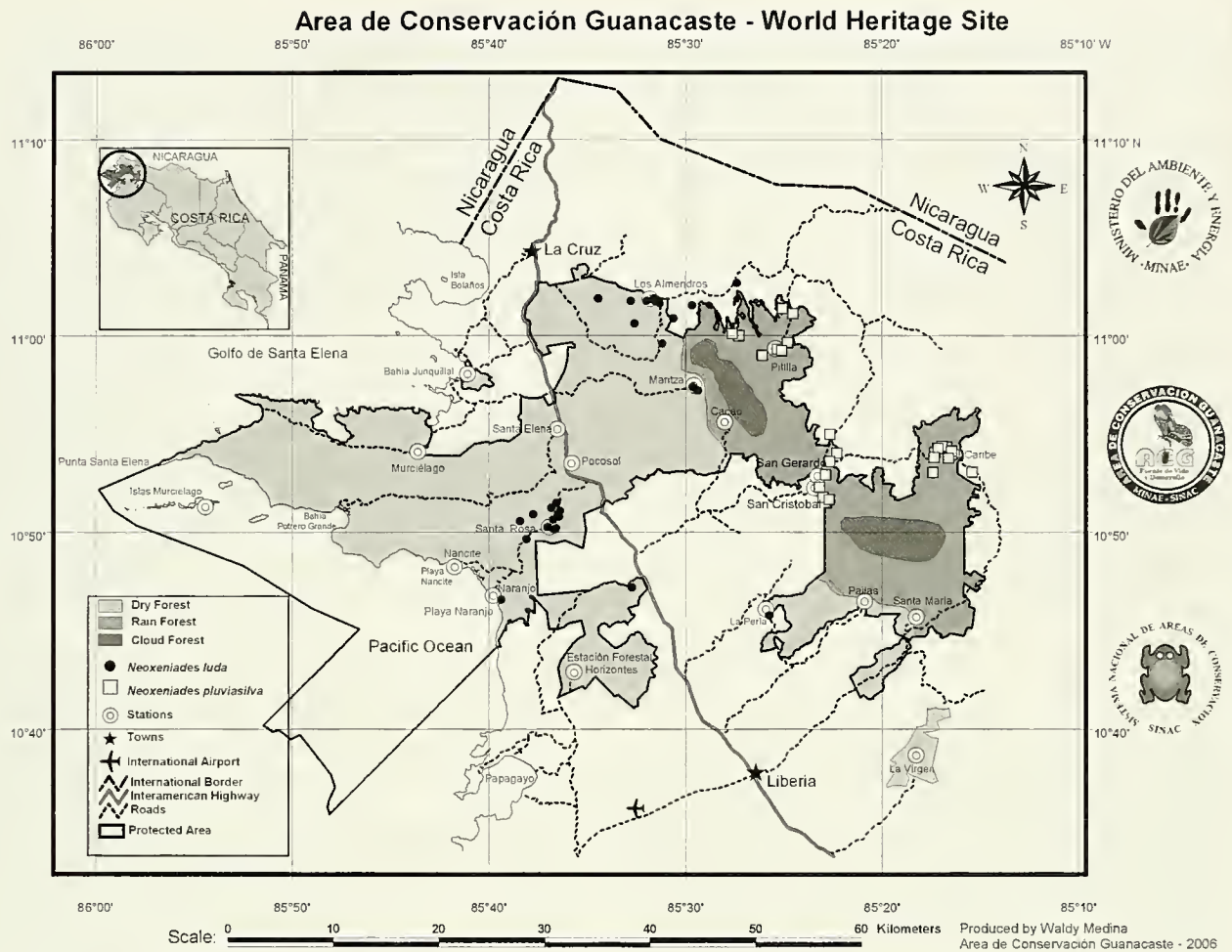


FIG. 3. Spatial distribution of *Neoxeniades luda* and *N. pluviasilva* in and near ACG.

Though clearly variations on a theme, the male genitalia of these two *Polyctor* species differ in striking ways. Despite substantial individual variation, almost every genitalic part differs interspecifically to at least some extent; but it is the highly asymmetric valvae that differ most (see Table 1 and cf. Figs. 28–33). Even the less elaborate female genitalia are notably distinct in the two species (Table 2).

Both species of *Cobalus*, which are predominantly brown, have a conspicuous white patch both dorsally and ventrally on a distal area of the hindwing. In ACG specimens, this patch is restricted to males of *C. fidicula* but expressed by both sexes of *C. virbius* (Figs. 8–11, 20–23), except for two females in which it is barely perceptible. Both species express it more fully ventrally than dorsally. In *C. fidicula* the patch stops before the outer margin so as to leave a narrow strip of dark brown ground color, ventrally the patch extends from mid space 1c to vein 6, and the white of the patch looks creamy on the ventral surface. In *C. virbius* the patch

reaches the outer margin, ventrally extends from the tornus to vein 6, and looks pure white on both wing surfaces. Lateral orange scaling—broad on the outer side of the palpus and narrow behind the eye—is bright in *C. fidicula* but just dully suggested, and only on the palpus, in *C. virbius*. *Cobalus fidicula* is a little larger than *C. virbius*, and its forewing hyaline spots are likewise larger.

The male genitalia (which are symmetric) differ in two obvious respects. The ventral distal division of the valva is longer and dorsally dentate in *C. fidicula* (cf. Figs. 35 and 37). The very broad uncus in dorsal view shows a pair of prominent lateral swellings in *C. virbius* (cf. Figs. 34 and 36).

Neoxeniades pluviasilva Burns, new species

(Figs. 3, 14, 15, 26, 27, 40, 41, 45, 46; Table 3)

Etymology. The species name, a noun in apposition, comes from the Latin *pluvia* for rain and *silva* for forest.

Diagnosis. This is a rainforest species whereas

TABLE 1. Major differences between male genitalia of *Polyctor cleta* and *P. polyctor*.

	<i>Polyctor cleta</i>	<i>Polyctor polyctor</i>
LEFT VALVA:		
Curved flap extending inward from dorsal margin	Narrower at base	Wider at base
Distal dorsal division	Not expanded; teeth finer	Expanded; teeth coarser
Distal ventral division	Bent sharply dorsad	Evenly curved dorsad
RIGHT VALVA:		
Distal dorsal division:		
Dentation on edge of	More distal; finer	More dorsal; coarser
SACCUS	More delicate; shorter to about gone	Heavier; longer
UNCUS, dorsally nr. origin	No longitudinal humps	Pair of longitudinal humps

N. luda is a species of the dry forest (Fig. 3). At a glance, *N. phuiasilva* is darker than *N. luda* and does not express a large, pale, outer marginal area on the ventral side of the hindwing nearly as well (cf. Figs. 26, 27 with 24, 25). In females of *N. phuiasilva*, the double hyaline cell spot of the forewing extensively overlaps the spot in space 2 whereas in *N. luda* females, this forewing cell spot overlaps the spot in space 2 little or not at all (cf. Figs. 15, 27 with 13, 25).

Description. Member, with *N. luda*, of mainly South American *N. scipio* species complex—treated by Evans (1955) as a polytypic species. This complex closely related to type species of *Neoxeniades*, *N. musarion* Hayward of Rio de Janeiro and Petrópolis, Brazil.

Facies: Brown ground color dark. Ventral brown ground color sexually dimorphic: warmer and rustier in male (Fig. 26); colder, with purplish gray overscaling, in female (Fig. 27). Sexual dimorphism also pronounced in size of forewing hyaline spots in space 2 and in cell: much larger in female than in male, with spots overlapping one another (cf. Figs. 15, 27 with Figs. 14, 26) [no such pronounced sexual dimorphisms in *N. luda* (Figs. 12, 13, 24, 25)]. Large, outer marginal, pale patch on ventral hindwing inconspicuous in male (Fig. 26) to all but nonexistent in female (Fig. 27) [patch always obvious in both sexes of *N. luda* (Figs. 24, 25)]. Dorsally, male always showing hyaline spot in space 1b just above mid vein 1, but spot almost always small (Fig. 14) to tiny. Female, more often than not, lacking this spot dorsally; but spot, when showing, usually tiny (Fig. 15). [In *N. luda*, male always with this spot, and spot usually well-expressed (Fig. 12); about two-thirds of females with this spot which, when present, more often small (Fig. 13) than tiny.]

Male genitalia: Symmetric; short anterior edge of slightly raised, dorsally dentate, distal end of valva curved somewhat cephalad in lateral view (Fig. 41) [about straight to curved somewhat caudad in lateral view (Fig. 39) in *N. luda*].

Female genitalia: Immediately anterior to papillae anales, sclerotized transverse plate of lamella postvaginalis with broad, rounded midventral elevation [narrower, more pointed in *N. luda*].

Type specimens: *Holotype:* male (Figs. 14, 26, 45, 46 [see arrow]), voucher code 06-SRNP-31674 (Janzen & Hallwachs 2006), Sendero Memos, Sector Pitilla, Area de Conservación Guanacaste, Costa Rica, 740 m, latitude 10.98171, longitude -85.42785. Deposition: National Museum of Natural History, Smithsonian Institution (USNM). Labeled (yellow): LEGS AWAY/FOR DNA. DNA barcode (658 bp) of holotype (coded MHAIHH575-06/06-SRNP-31674) (*Neoxeniades phuiasilva*):

AACTTTATACTTTATTTTTGGAATTTGAGCAGGAATATTAGG
AACTTCATTAAGTTTATTAATCCGTACAGAATTTGGGAAATCCAG
GATTTTTAATTGGAAATGATCAAATTTCAATACTATTGTACAG
CTCATGCATTTATTATAATTTTTTTTATAGTTATACCTATTATAAT
TGGAGGATTTGGAAATGATTAGTACCTTTAATATTAGGAGCT
CCAGATATAGCTTTCCCTCGATTAATAATAAGATTTTGATTA
TTACCTCCTTCTTTAATACTTTAATTTAATAAGAAGAAATTTGAGA
AAATGGAGCTGGCACTGGATGAAGCTTTTATCCCCCTCTTTC
CTTAACATTTGCTCATCAAGGATCATCTGTAGATTTAGCAATCT
TCTCACTCCATCTAGCTGGAATTTTCATCTATTTTAGGAGCTATT
AATTTTATACCACAAATTAATAATGCGAATAAAAATTTTATCTT
TTGATCAAATATCTTATTCTGTGATCTGTTGGTATFACTGCT
TTACTTTTACTCTTATCTCTACCAGTCTTAGCTGGAGTATTAC
AATATTACTTACTGACCGAAATCTTAATACTTTCTTTTTTCGACC
CAGCAGGAGGAGGAGATCTTATTTATATCAACATTTTATT

Paratypes: 14 males, 19 females, ACG, Costa Rica (USNM).

The lone synonym of *N. luda* is *Proteides hundurensis* Mabille. Mabille's (1891) original description of one female from Honduras clearly applies to *N. luda*.

Foodplants: Larval foodplants of this rainforest skipper at least eight species in five genera (one introduced) of Bromeliaceae (Table 3) [no known overlap in foodplant use at species level with dry-forest, sister species *N. luda*; but one overlap at genus level].

Larval foodplants (Table 3). Foodplants do not distinguish sister species of the pyrgine genus *Polyctor*: both of these skippers eat one and the same plant species in each of three genera (*Allenanthus*, *Coutarea*, and *Exostema*) of the family Rubiaceae. Nevertheless, *P. cleta* and *P. polyctor* use these plants at very different frequencies. Although this may reflect different preferences of the skippers, it more likely stems from the different ecologic distributions of the foodplants: *Coutarea hexandra* occurs at low frequency in both rain and dry forest, *A. erythrocarpus* is a rainforest plant

TABLE 2. Major differences between female genitalia of *Polyctor cleta* and *P. polyctor*.

	<i>Polyctor cleta</i>	<i>Polyctor polyctor</i>
Sclerotized sterigmal area	Smaller	Larger
Overall asymmetry	Not extreme	Pronounced
Sclerotized flap on right side of sterigma	Absent or small	Very large
Sclerotized ostium bursae	Conspicuously projecting (tubelike) from surface of sterigma	Relatively flush with surface of sterigma

TABLE 3. Larval foodplants of two species of *Polyctor*, *Cobalus*, and *Neoxeniades* in Area de Conservación Guanacaste, northwestern Costa Rica; the number of rearing records for each plant species is given (source, Janzen & Hallwachs 2006).

<i>Polyctor cleta</i>		
Rubiaceae		
<i>Allenanthus erythrocarpus</i>		9
<i>Coutarea hexandra</i>		7
<i>Exostema caribaeum</i>		1
<i>Exostema mexicanum</i>		124
<i>Polyctor polyctor</i>		
Rubiaceae		
<i>Allenanthus erythrocarpus</i>		44
<i>Coutarea hexandra</i>		166
<i>Exostema mexicanum</i>		1
<i>Cobalus virbius</i>		
Arecaceae		
<i>Acrocomia aculeata</i>		7
<i>Bactris guineensis</i>		5
<i>Cobalus fidicula</i>		
Arecaceae		
<i>Astrocaryum alatum</i>		25
<i>Bactris gasipaes</i> (introduced)		5
<i>Bactris gracilior</i>		6
<i>Bactris hondurensis</i>		10
<i>Chamaedorea dammeriana</i>		1
<i>Chamaedorea pinnatifrons</i>		1
<i>Cryosophila warszewiczii</i>		1
<i>Prestoea decurrens</i>		1
<i>Neoxeniades luda</i>		
Bromeliaceae		
<i>Aechmea bracteata</i>		4
<i>Aechmea magdalenae</i>		30
<i>Bromelia pinguin</i>		138
<i>Neoxeniades pluviasilva</i>		
Bromeliaceae		
<i>Aechmea pubescens</i>		12
<i>Ananas comosus</i> (introduced)		3
<i>Guzmania desautelsii</i>		3
<i>Guzmania donnellsmithii</i>		12
<i>Guzmania nicaraguensis</i>		1
<i>Pitcairnia arcuata</i>		1
<i>Pitcairnia atrorubens</i>		4
<i>Vriesea gladioliflora</i>		19

(used by *P. cleta* at the very edge of the dry forest/rain forest ecotone), and *E. mexicanum* is a common dry-forest plant (used once by a *P. polyctor* that apparently strayed some 15 km from rain forest).

Conversely, foodplants do seem to distinguish the species of the species pairs in the hesperiine genera *Cobalus* and *Neoxeniades*. In each pair, the rainforest skipper feeds on more species than its dry-forest counterpart. *Cobalus virbius* eats two plant species in two genera, and *C. fidicula* eight plant species in five genera, of the family Arecaceae (palms). *Neoxeniades luda* eats three plant species in two genera, and *N. pluviasilva* eight plant species in five genera, of the family Bromeliaceae (bromeliads). The species in each skipper pair share no foodplant species and just one genus. However, these considerable differences in diet may have little taxonomic significance. Palms and bromeliads are far more diverse in rain forest than they are in dry forest, so that the rainforest skippers have a wider choice. Were either species of a pair to invade the other's ecosystem, it might well find its relative's foodplants acceptable. This conjecture is somewhat weakened by the fact that the rainforest skippers, though polyphagous, still restrict their diet to fewer species of palms and bromeliads than are available to them; and even one dry-forest species is somewhat choosy. To illustrate, the ACG dry forest provides only two species of palms for *C. virbius*, both of which it eats, whereas the rain forest offers >10 species of palms beyond the eight so far recorded for *C. fidicula*. The dry-forest skipper *N. luda* eats the three terrestrial bromeliad species, but not the epiphytic ones, available to it, whereas *N. pluviasilva* eats both kinds of bromeliads, but seems nevertheless to ignore many of the epiphytic species at hand.

DNA barcodes (Figs. 42–46). For our barcoding methods, see Hajibabaei *et al.* (2006:971). We determined sequence divergences among individuals in each species pair (as well as in a related outgroup species) by means of the Kimura-2-Parameter (K2P) distance model (Kimura 1980), and then showed these divergences in neighbor-joining (NJ) trees (Saitou & Nei 1987). A paper in preparation will deposit in GenBank all of the sequences used here (along with thousands more for hundreds of species in various lepidopteran families, including Hesperidae). All voucher specimens have been deposited in the National Museum of Natural History, Smithsonian Institution.

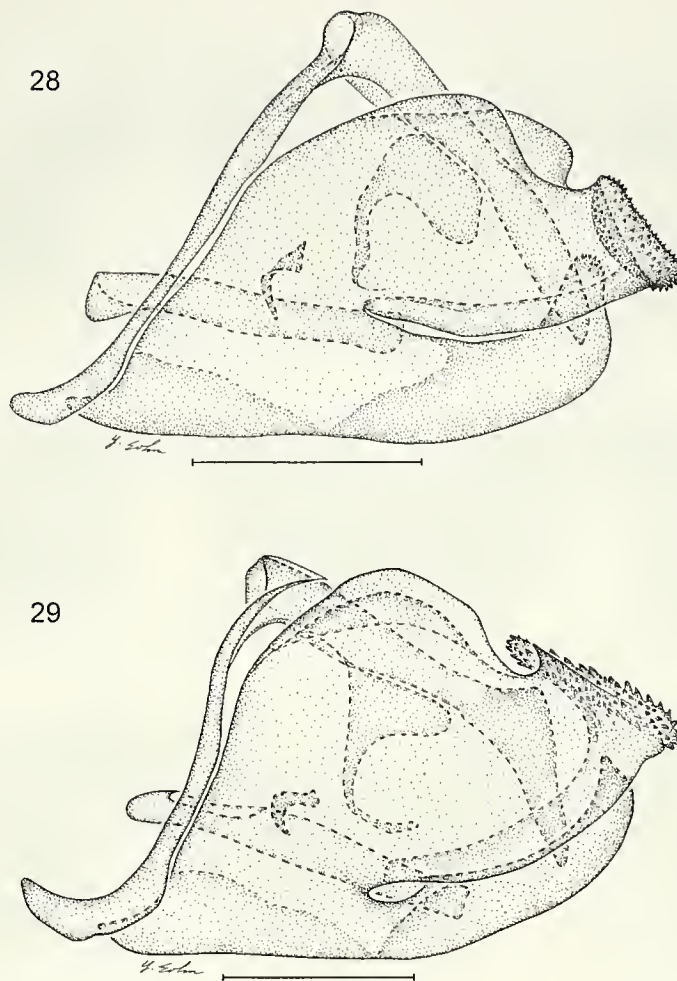
Since we could not discriminate between the species in each pair during our early barcoding efforts, we greatly increased sample sizes; but we still included some COI sequences that were too short to qualify as legitimate barcodes. Two of the resulting NJ trees



FIGS. 4–15. Reared adults in dorsal view of *Polyctor*, *Cobalus*, and *Neoxeniades* from ACG, Costa Rica (specimens in USNM). Males even-numbered, females odd-numbered. Wingspan and voucher code given for each specimen. **4, 5**, *P. cleta*: 32 mm, 02-SRNP-322S5; 40 mm, 06-SRNP-S69. **6, 7**, *P. polyctor*: 35 mm, 05-SRNP-4224S; 34 mm, 03-SRNP-9639. **8, 9**, *C. virbius*: 32 mm, 92-SRNP-6215.1; 32 mm, 92-SRNP-46. **10, 11**, *C. fidicula*: 39 mm, 05-SRNP-2306S; 36 mm, 04-SRNP-3240S. **12, 13**, *N. luda*: 49 mm, 01-SRNP-11651; 53 mm, 03-SRNP-38341. **14, 15**, *N. pluviasilva*: 43 mm, 06-SRNP-31674; 52 mm, 05-SRNP-22927.



FIGS. 16–27. Reared adults in ventral view of *Polyctor*, *Cobalus*, and *Neoxeniades* from ACG, Costa Rica (specimens in USNM). Males even-numbered, females odd-numbered. Same specimens in same sequence as in Figs. 4–15. 16, 17, *P. cleta*. 18, 19, *P. polyctor*. 20, 21, *C. virbius*. 22, 23, *C. fidicula*. 24, 25, *N. luda*. 26, 27, *N. phviasilva*.



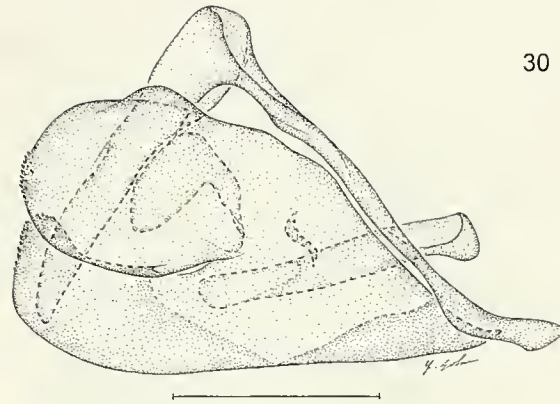
FIGS. 28, 29. Asymmetric male genitalia in left lateral view of two species of *Polyctor* from ACG, Costa Rica (USNM), scale = 1.0 mm. 28, *P. cleta*, genitalia dissection code X-6165, voucher code 03-SRNP-30880. 29, *P. polyctor*, X-6159, 02-SRNP-7128.

nearly separated the two species of *Polyctor* (Fig. 42) and those of *Cobalus* (Fig. 44). However, the third tree appreciably intermixed the two species of *Neoxeniades* (Fig. 45). The short sequences lacked diagnostic sites and therefore compromised the NJ analysis. Subsequent exclusion of short sequences resulted in clear species separation (Figs. 43, 46).

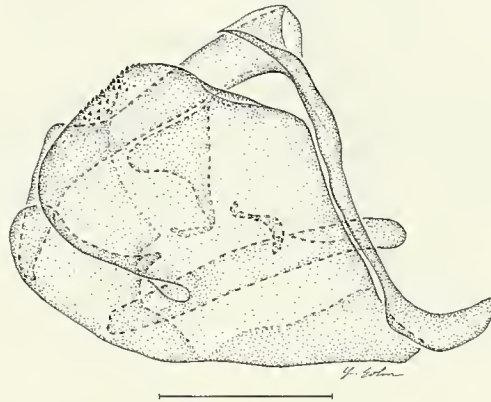
Close examination of the barcode nucleotides showed that the two species of *Polyctor* consistently differ at three nucleotide positions (610, 616, and 625), and the two species of *Neoxeniades*, at one (115). Similarly, the two species of *Cobalus* differ in one nucleotide (at position 181), except for two females of *C. virbius* (04-SRNP-21798 and 06-SRNP-22664) whose “diagnostic” nucleotide is the same as that of the *C. fidicula* specimens. The two females of *C. virbius* whose

barcodes match those of *C. fidicula* are not the two females of *C. virbius* (noted above, under “Morphologic differences” [with voucher codes 92-SRNP-340 and 06-SRNP-13344]) whose hindwing facies approaches that of *C. fidicula* females. Because, in each pair of skipper species, the interspecific nucleotide differences are very few (compared with many species of skippers previously examined), full-length, high-quality barcode sequences (~650 bp) are critical for distinguishing the species in each pair. Of course, characters of this kind—like many others—may vary geographically.

The levels at which these species are distinguished is so low that, in many other circumstances, their differences could easily qualify as nothing more than individual variation. It follows that the designation of some percentage or degree of divergence as a point

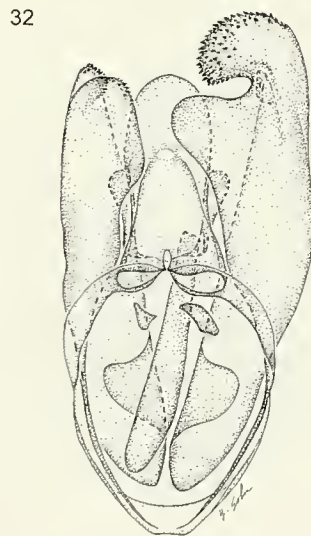


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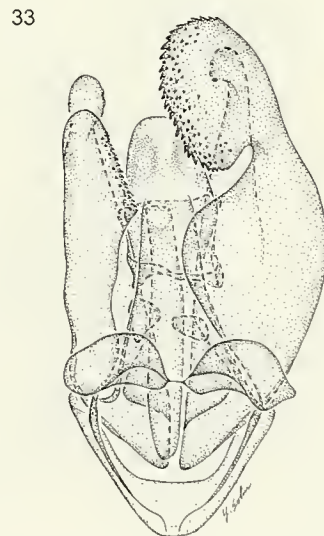


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FIGS. 30, 31. Asymmetric male genitalia in right lateral view of two species of *Polycitor* from ACG, Costa Rica (USNM), scale = 1.0 mm. **30**, *P. cleta*, X-6165, 03-SRNP-30880. **31**, *P. polycitor*, X-6159, 02-SRNP-712S.

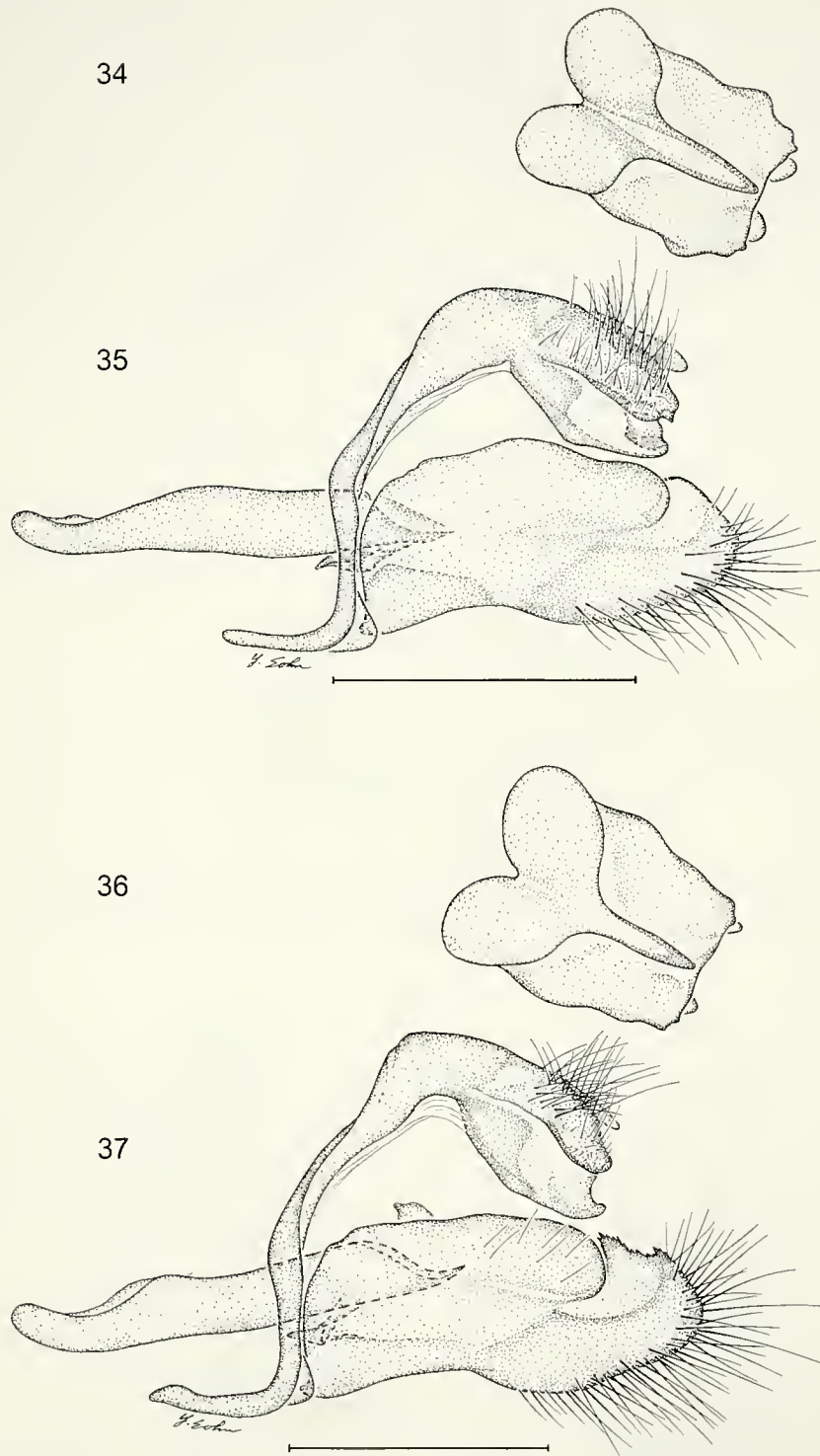


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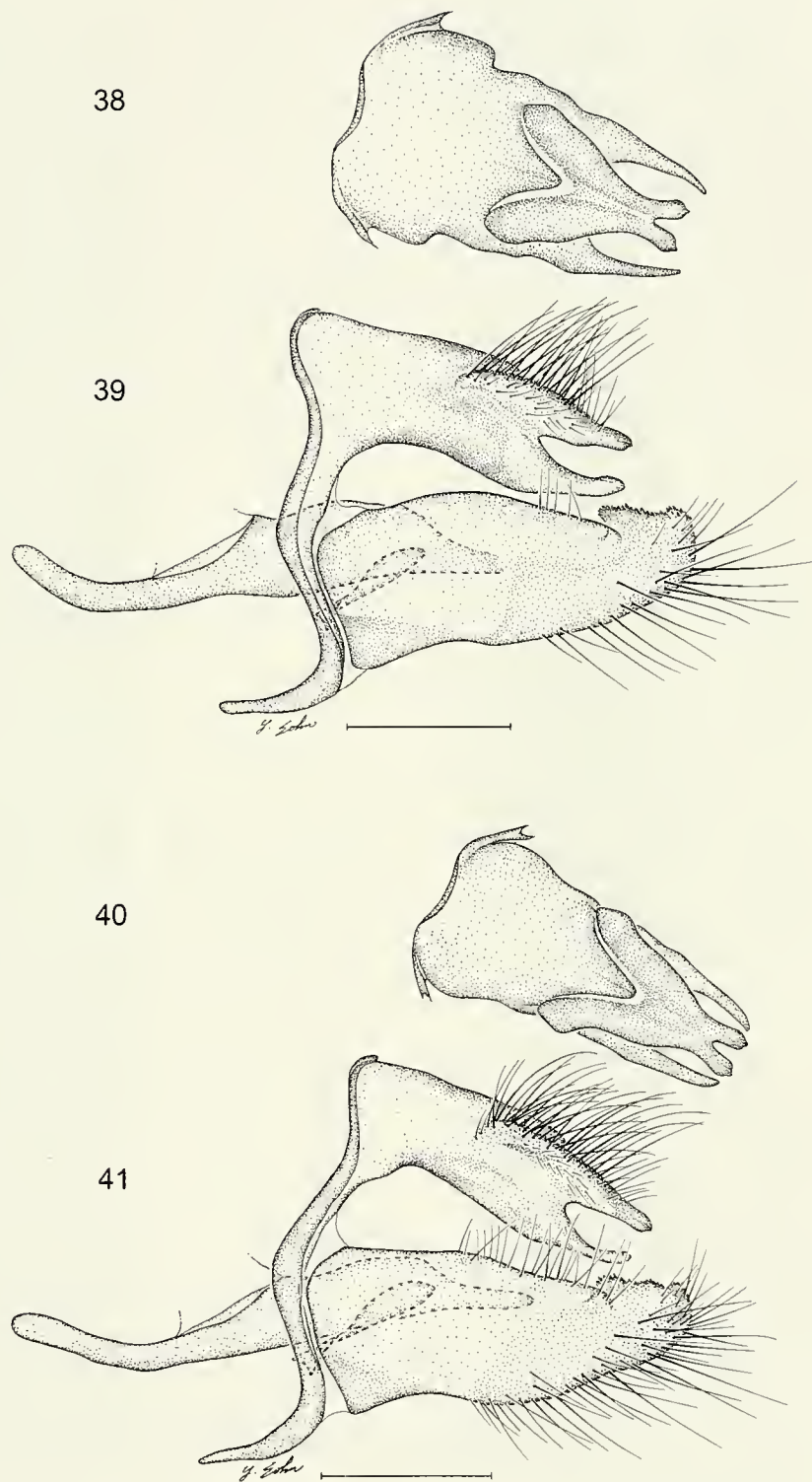


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FIGS. 32, 33. Asymmetric male genitalia in dorsal view of two species of *Polycitor* from ACG, Costa Rica (USNM), scale = 1.0 mm. **32**, *P. cleta*, X-6165, 03-SRNP-30880. **33**, *P. polycitor*, X-6159, 02-SRNP-712S.



FIGS. 34-37. Male genitalia of two species of *Cobalus* from ACG, Costa Rica (USNM), scale = 1.0 mm. **34, 35**, *C. virbius*, X-5S73, 99-SRNP-6213. **36, 37**, *C. fidicula*, X-5S76, 99-SRNP-5803. **34, 36**, Tegumen, uncus, and gnathos in dorsal view. **35, 37**, Complete genitalia in left lateral view.



FIGS. 38–41. Male genitalia of two species of *Neoxeniades* from ACG, Costa Rica (USNM), scale = 1.0 mm. 38, 39, *N. luda*, X-6372, 95-SRNP-10736. 40, 41, *N. pluviasilva*, X-5799, 00-SRNP-2211. 38, 40, Tegumen, uncus, and gnathos in dorsal view. 39, 41, Complete genitalia in left lateral view.



FIGS. 42, 43. Neighbor-joining (NJ) trees based on Kimura-2-Parameter (K2P) distances for cytochrome *c* oxidase I (COI) of *Polyctor* from ACG, Costa Rica. Outgroup, *P. enops*. Rearing voucher code and sequence length given for each individual. To find the larval foodplant of any individual, enter its voucher code in the ACG database (Janzen & Hallwachs 2006). **42**, 19 *P. cleta* and 44 *P. polyctor* with various COI sequence lengths. **43**, 17 *P. cleta* and 35 *P. polyctor* with COI barcodes of sufficient lengths (approaching or exceeding 650 base pairs) for distinguishing these species.

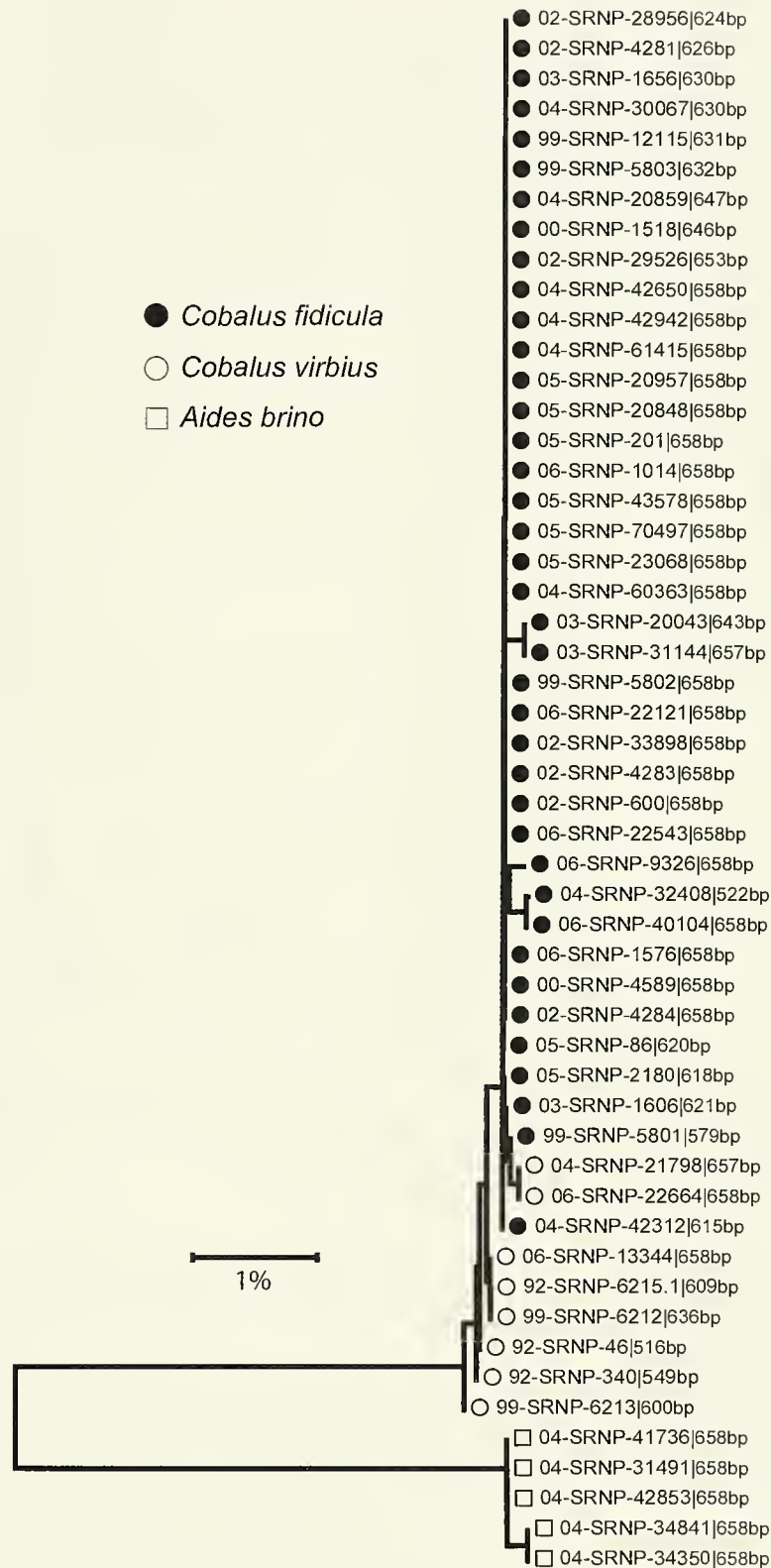


FIG. 44. NJ tree based on K2P distances for COI of 8 *Cobalus virbius* and 39 *C. fidicula* from ACG, Costa Rica. Outgroup, *Aedes brino*. Rearing voucher code and sequence length given for each individual.



FIGS. 45, 46. NJ trees based on K2P distances for COI of *Neoxeniades* from ACG, Costa Rica. Outgroup, *N. BURNS04* (an undescribed species). Rearing voucher code and sequence length given for each individual. Arrow indicates holotype. **45**, 13 *N. luda* and 24 *N. pluviasilva* with various COI sequence lengths. **46**, 7 *N. luda* and 12 *N. pluviasilva* with COI barcodes of sufficient lengths for distinguishing these species.

below which individuals should be considered conspecific is unrealistic (even though many taxonomists have done so, in various contexts, for a great many years). Speciation is not tidy.

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